Free amino acids, urea and ammonium ion contents for submerged wine vinegar production: influence of loading rate and air-flow rate

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Abstract

The nitrogen source for acetic acid bacteria is important during the vinegar making process. There can be great variation in the final result according to the specific source, the total nitrogen availability and the operational conditions. These bacteria use L-proline, L-leucine and ammonium ion as their main source of nitrogen from white wine. The effect of loading and air-flow rates on the changes in amino acids, urea and ammonium ion contents have been studied for a semi-batch submerged wine vinegar controlled production. Experiments were carried out in a Frings 8L fermenter working in a semi-batch mode. Amino acid contents were determined from their dansyl derivatives on an HPLC furnished with a C18 reversed-phase column. Urea and ammonium ion contents were quantified with an enzymatic kit. Specific nitrogen consumption is given for 25 amino acids and ammonium ion. In addition, profiles for main system variables as well as the three main nitrogen sources (ammonium ion, L-leucine and L-proline) are given. Type of loading and air-flow rates seemed to have a strong impact on the consumption of the nitrogen compounds tested. An increased loading rate and decreased air-flow rate resulted in greater overall consumption of available nitrogen due to different causes. Nitrogen requirement of the bacteria is proportional to the time spent in the acetification process. An acetification procedure involving relatively sudden changes in the fermentation medium may be desirable in order to reduce the formation of urea.

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Introduction

It is well known that acetic acid bacteria (AAB) are industrially used to produce vinegar.[1-3] In this process, the bacteria use ethanol as a carbon and energy substrate and free amino acids and ammonium ion as main nitrogen sources.[4] The amino acids are also intermediates of other compounds that could influence product quality. Though AAB are able to synthetize amino acids from ammonium ion, a minimum presence of these compounds is desirable to facilitate their action. Adequate amino acids content in the acetification raw medium is not always guaranteed because wine vinegar results from two fermentation processes. First, an alcoholic fermentation by yeast followed by ethanol oxidation by AAB. The previous yeast activity as well as some fining wine treatments may decrease nitrogen availability in the medium compromising the next stage.[4-6] L-proline, L-methionine, L-leucine, L-ornithine and ammonium ion may together account for approximately 70% of the total nitrogen content in the wine[7] (mainly L-proline with approximately 40%). The high L-proline content can be explained by considering its high concentration in grape must,[4] as well as both the limitation of molecular oxygen during the alcoholic fermentation that prevents its degradation[6] and the fact that L-proline metabolized very little by yeast.[8] On the other hand, the presence of L-leucine can be explained by taking into account the fact that yeasts release this amino acid during alcoholic fermentation.[4]

So far, the analysis of amino acid content has been applied to the characterization of wine vinegar, to study the chemical and biochemical transformations that take place during ageing, to explore the requirements of AAB strains for these compounds, and to study the differences in the pattern of amino acid consumption between surface and submerged acetifications.[9-13]

Despite its importance for the process, the nitrogen source variation during acetic acid fermentation in wine is poorly documented. The authors, therefore, carried out a study[7] to examine changes in free amino acids, urea and ammonium ion during acetification in a semi-batch culture of AAB. The work aimed to evaluate possible nitrogen

limitations in the raw medium, the amino acid changes throughout the cycle, as well as its relationship with other variables in the system. The bacteria were found to use L-proline, L-leucine and ammonium ion as main sources of nitrogen. Also, the profiles for amino acid concentrations were very similar in all the cases and were related to changes in cell concentrations. In addition, it was very interesting to find urea in the vinegar but not in the wine. Since urea is a precursor for ethyl carbamate, a carcinogenic compound found in fermented beverages, its production must be reduced as much as possible.[4,14,15] The present study was, therefore, carried out to examine the changes in free amino acids, urea and ammonium ion contents under different operational conditions for a semi-batch submerged wine vinegar production in order to evaluate the influence of two very important operational variables: loading rate and air-flow rate.

Materials and Methods

Microorganisms

The original inoculum used was obtained from a fully operational industrial fermenter working with wine as previously described.[7]

Medium and acetification conditions

A white wine from the Montilla-Moriles region (southern Spain) with an ethanol concentration of 11.5±0.5 %(v/v) and an initial acidity of 0.4% (w/v), expressed as g of acetic acid×100 mL^{-1} of medium was used.

Experiments were carried out in a Frings 8L fermenter working in a semi-batch mode. For a specific type of wine, the operational variables that can usually be modified are: temperature, final ethanol concentration, unloading percentage of the medium, loading flow rate and air-flow rate. This study compared three different operational conditions. In all cases, temperate (31°C), final ethanol concentration (0.5±0.1 %v/v), final acidity (10.0±0.2% w/v) and unloading percentage of the medium (75%) were the same for all experiments, but loading and air-flow rates were modified.

The bioreactor was fully automated. Loading, unloading, control and monitoring operations were performed via a previously programmed computer. A semi-continuous operational mode was used. Therefore, once it was partially unloaded, a new cycle was started by adding fresh wine. The experimental process was repeated at least four times for each case.

Statistical analysis

Amino acid, urea and ammonium ion concentrations were determined by previously passing the samples through 0.45 μm Millipore filters and adjusting pH to 7.5 with NaOH, with provision for the dilution factor.

Urea and ammonium ion in the medium were quantified with an enzymatic kit from Boehringer-Mannheim/R-Biopharm (Germany).

Amino acid contents were determined according to Botella *et al.*[16] vinegar samples (2 mL) were centrifuged (6000 g, 5 min) and filtered (0.22 μm Millipore filter). The chromatographic apparatus consisted of a Spectra-Physics P200 HPLC including a SP 8450UV-VIS detector for measuring the absorbance at 254 nm. The column used was a 150×4.6 mm, 5 μm C18 Spherisorb ODS resin from Tracer Analytical (Barcelona, Spain). The column was thermostated at 25°C. For derivatization, a 0.1 mL filtered sample was dissolved in lithium carbonate buffer (40 mM pH 9.5, with HCl), with L-Norleucine as internal standard (5 mM). Then, Dns-Cl dissolved in acetonitrile (14.8 mM) at 28°C was added. The reaction was terminated by adding 100 μL of 2% triethanolamine after 1 h. Twenty microliters of this was injected into the HPLC column. Samples were protected from light during all the operations. For the elution process, a system of binary gradients consisting of the solvent A of methanol and the solvent B of a watery solution of acetic glacial acid to 0.6 % (v/v) and triethylamine to 0.008 % (v/v) was used (Table 1).

Table 1. Elution gradient for the HPLC separation of amino acids.

Time (min)	0.0	20.0	25.0	50.0	55.0	65.0	70.0	75.0	76.0	86.0
A (%)	30	30	40	50	50	65	75	75	30	30
B (%)	70	70	60	50	50	35	25	25	70	70
Flow rate	1.0	1.0	1.0	1.5	2.0	2.0	2.0	2.0	1.0	1.0

(A) Methanol. (B) Watery solution of acetic glacial acid to 0.6 % (v/v) and triethylamine to 0.008 % (v/v).

Amino acids were identified by comparing their relative retention times with those for standards obtained from Sigma-Aldrich (Barcelona, Spain). Data were collected and analyzed using Biochrom 2000 computer software.

Acidity was determined by acid-base titration, an online probe Alcosens (Heinrich Frings GmbH & Co. KG, Bonn, Germany) and a differential pressure sensor (Yokogawa Iberia S.A., Madrid, Spain) were used for ethanol and volume determination, respectively.[17]

The oxygen concentration was continuously measured using a dissolved oxygen sensor (SM31) and a proper converter (DO402G), both from Yokogawa (*http://www.yokogawa.com/*).

Results and Discussion

Figures 1, 2 and 3 show the experimental profiles for the main system variables: cell, ethanol and oxygen concentrations as well as volume and acidity of the medium.

Figure 4 shows the variation of the available nitrogen in ammonium, urea and free amino acids in the wine and the resulting vinegars according to the different fermentation conditions.

A correct interpretation of the results requires consideration of the fact that the semi-continuous working method used involves repeating a series of cycles. According to this, once 75% of the volume of the medium has been unloaded when ethanol concentration has reached 0.5% (v/v), a loading stage lasting as long as is necessary to reach the fermenter's full operational volume is started. Changes in the concentrations of the different variables during the loading stage are due not only to cellular activity, but also to the fresh medium added to the fermenter (*i.e.* to compound addition and a dilution effect). As can be seen from Figures 1-3, the ethanol concentration rises and the simultaneous dilution effect reduces the cell concentration and acidity of the medium during the loading process. Obviously, the variation profiles depend on the particular operating conditions. The ethanol concentration typically increases, and the acidity and cell concentration decrease, during the latter stage.

The results shown in Figures 1-3 were subjected to a procedure previously reported by the authors[18] to assess the mean acetification rate and productivity of each experiment. The results are shown in Table 2. As can be seen, there were no appreciable differences in either variable between V1 and V2. Therefore, the loading rate seems not to have had any substantial influence under the experimental conditions used. On the other hand, the loading rate considerably influenced the environmental conditions for bacterial growth during the loading process in experiment V2. As can be seen from Figures 1 and 2, the loading stage took approximately 10 h to complete in V1 but only 1.5 h in V2. There was, therefore, a difference in the variation of ethanol and acidity in

the medium that can result in adverse circumstances for bacteria, particularly if one considers the sensitivity of acetic bacteria to ethanol an acetic acid.[19-21] Figure 4 illustrates the potential effect of changes in the operational variables through the variation of the amount of available nitrogen in the raw material and end products. As can be seen from Table 3, an increased loading rate (V2) resulted in greater overall consumption of available nitrogen. An abrupt change in the environmental conditions, therefore, seems to lead to a stress situation in which cells must use greater amounts of nitrogen to adapt to the medium. The process by which acetic bacteria adapt to these drastic conditions has already been described.[22]

The greatest consumption of amino acids present in the original wine were for L-proline, L-methionine and L-ornithine; percentages of consumption are shown in Table 3. Marked differences in the use of nitrogen can be seen between V1 and V2. In contrast, ammonium ion was consumed to a greater extent at the lower loading rate (V1). Apparently, cells in the medium tend to use free amino acids preferentially over ammonium ion in response to an abrupt change in the environmental conditions; in this situation, cells can adapt more easily by using existing amino acids than by synthesizing them *de novo*, which would take longer. Therefore, a different method of regulating amino acid synthesis was adopted according to the environmental conditions.

Table 2. Operational variables used, time of cycle, acetification rate and acetic acid productivity in the three experiments (V1, V2 and V3).

Experiment	V1	V2	V3
Constant air flow-rate ($L \cdot h^{-1}$)	60	60	30
Constant loading rate ($L \cdot min^{-1}$)	0.01	0.06	0.06
Cycle duration (h)	46.1±0.9	39.1±1.9	65.5±5.5
Mean acetification rate (g acetic acid$\cdot L^{-1} \cdot h^{-1}$)	0.17±0.01	0.16±0.01	0.10±0.01
Acetic acid productivity (g acetic acid$\cdot h^{-1}$)	12.8±0.3	12.9±0.7	8.0±0.7

Table 3. Percentages of main nitrogen consumptions by the bacteria in the V1, V2 and V3 experiments.

Amino acid	Exp. V1 (%)	Exp. V2 (%)	Exp. V3 (%)
L-Proline	25.7	59.8	69.4
L-Methionine	15.4	85.5	96.9
L-Ornithine	30.8	69.6	52.1
Ammonium ion	87.7	62.6	78.6
Total assimilable nitrogen	34.1	49.8	59.5

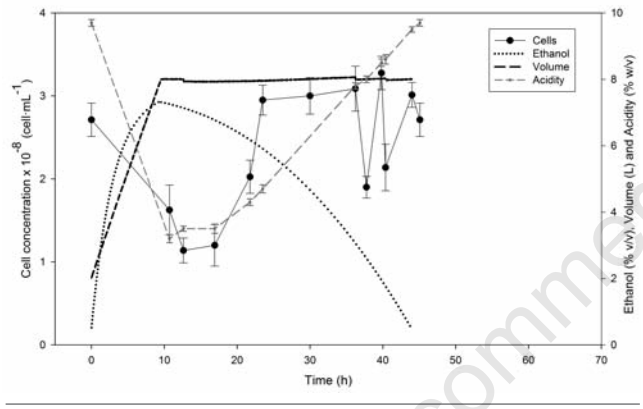

Figure 1. Variation in the concentrations of cells, ethanol, volume and acidity of the medium during the V1 acetification cycle. Bars represent standard deviations (SD). The corresponding mean SD for ethanol and volume were approximately 3% and 2%, respectively.

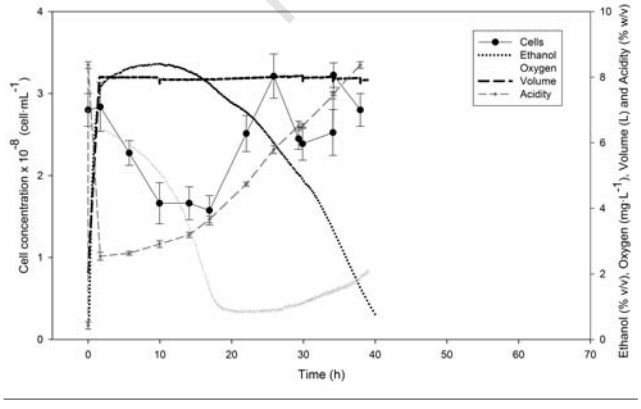

Figure 2. Variation in the concentrations of cells, ethanol, oxygen, volume and acidity of the medium during the V2 acetification cycle. Bars represent standard deviations (SD). The corresponding mean SD for ethanol, oxygen and volume were approximately 3%, 2% and 2%, respectively.

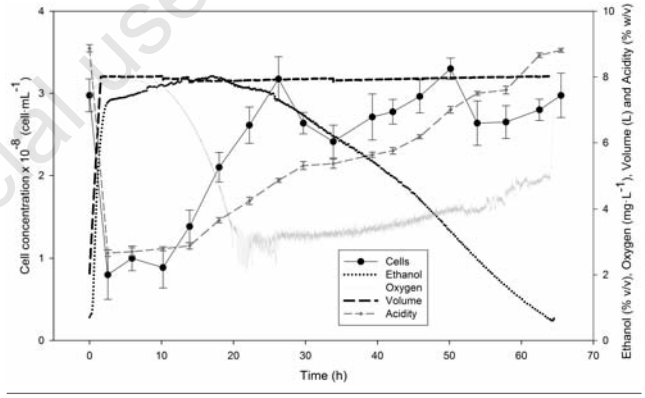

Figure 3. Variation in the concentrations of cells, ethanol, oxygen, volume and acidity of the medium, during the V3 acetification cycle. Bars represent standard deviations (SD). The corresponding mean SD for ethanol, oxygen and volume were approximately 3%, 2% and 2%, respectively.

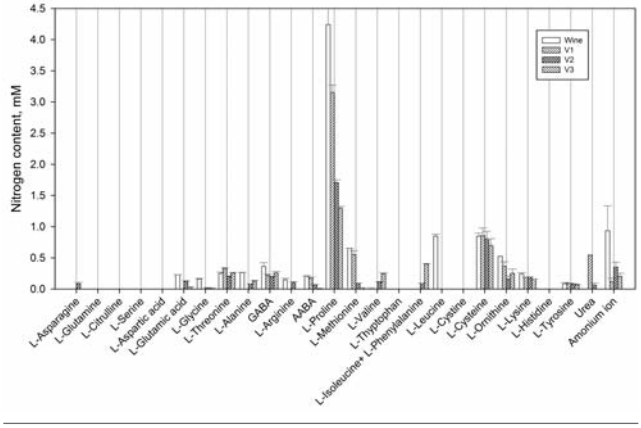

Figure 4. Mean concentrations in nitrogen (mM) from ammonium ion, urea and free amino acids of the starting wine and the obtained vinegars (V1, V2 and V3). Bars represent standard deviations (SD).

Thus, under mild conditions, cells probably use ammonium ion to produce amino acids that are partly stored in the medium as a reserve for more adverse future conditions.

As expected, changing the air flow-rate in addition to the loading rate (experiment V3) considerably reduced the mean acetification rate and productivity (Table 2). Also, it led to substantial differences in the total amount of nitrogen used by the cells in relation to that initially present in the wine; thus, nitrogen consumption rose to approximatley 60% which was nearly twice that seen in experiment V1 (Table 3). The greater length of experiment V3 probably resulted in greater consumption of some compounds in cell maintenance activities and, as can be seen, such activities involved an increased use of available nitrogen sources. Under these conditions, L-proline continued to supply most of the nitrogen used. Other authors[12] had previously found the nitrogen requirements of acetic bacteria to be proportional to the acetification time. Also, since L-proline, L-leucine and ammonium ion in combination supplied almost 80% of all nitrogen, Figure 5 shows the variation in the amount of nitrogen in the form of these compounds during the fermentation cycle. As can be seen, the results of experiments V2 and V3 were consistent with those previously reported by the authors[7] for experiment V1. This seems to confirm a relationship between the observed oscillations in cell concentrations and the contents in amino acids; this was especially apparent in experiment V3 by virtue of its greater duration. These oscillations are suggestive of the presence of growth and autolysis as a mechanism for cells to adapt to experimental conditions not favoring exponential cell growth. Under these conditions, the above-described situation can be a result of a high concentration of ethanol or acetic acid. It has been suggested that autolysis in bacteria represents an apoptosis mechanism intended to ensure persistence of a microbial population.[23] Other authors have also reported diauxic growth in acetic bacteria.[22]

Figure 5 compares the profiles of the three compounds as a function of the feed flow-rate during the loading phase and the air flow-rate. As can be seen, whereas the air flow-rate resulted in no significant differences between the profiles except in cycle duration, changing the flow-rate of fresh medium during the loading stage had a marked effect. Thus, the bacteria seemingly tended to use ammonium ion in preference over leucine and proline as their nitrogen source.

Figure 4 also shows that some compounds, including L-valine, L-isoleucine and L-phenylalanine (both jointly) and urea increased during the process. The increase in urea was especially interesting since this compound is known to be a precursor for ethylcarbamate.[4,14,15] Ethylcarbamate is a carcinogen typically found in fermented beverages; its formation should, therefore, be avoided as much as possible. To this end, it would be desirable, if at all possible, to use operating conditions that hinder the formation of urea. The results show that using a low loading rate (experiment V1) led to an increased formation of urea; therefore, it seems to be preferable to use a higher loading rate.

Our results suggest that obtaining vinegar from wines containing little nitrogen requires a careful procedural design since prolonged acetification can lead to depletion of available nitrogen. Also, an acetification procedure involving relatively abrupt changes in the fermentation medium may be desirable in order to reduce the formation of urea. Among the three different experimental conditions, experiment V2 seems to be the best since high productivity is obtained with a low level of urea formation.

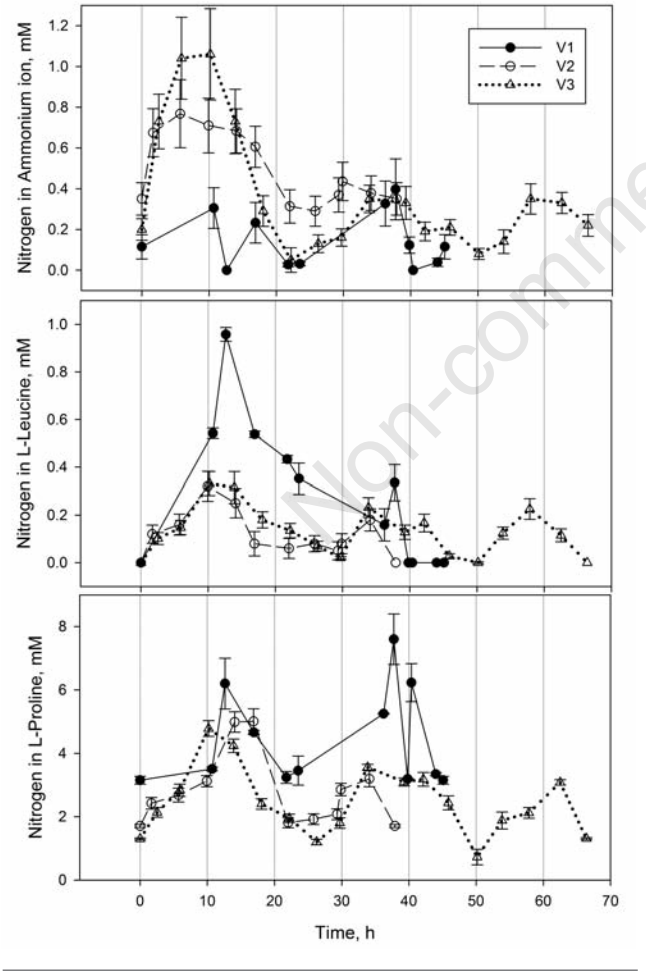

Figure 5. Variation in the nitrogen in L-proline, L-leucine and ammonium ion during the V1, V2 and V3 acetification cycles. Bars represent standard deviations (SD).

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